

Addendum added 22 May 2012

Response to Comment on “Fossilized Nuclei and Germination Structures Identify Ediacaran ‘Animal Embryos’ as Encysting Protists”

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The objections of Xiao *et al.* to our reinterpretation are based on incorrect assumptions. The lack of nanocrystals lining the nuclear membrane is consistent with membrane fossilization, and nucleus volume through development is correlated to cytoplasm volume and fully consistent with sizes of eukaryote nuclei. Identical envelope structure unites the developmental stages of the fossils, and 2ⁿ cleavage and Y-shaped junctions are holozoan symplesiomorphies.

Xiao *et al.* (1) claim that our reassessment (2) of previously interpreted “animal embryos” (3) rests on two flawed interpretations: The structures we identify as nuclei are not nuclei, and the proposed life cycle conflates two distinct organisms. Both claims are flawed.

We identified the nuclei based on a number of morphological features involving recurrence, position within cells, shape, volumetric relationship between nuclei and cells, and evidence of mitotic division. In all these features, the structures conform to typical cell nuclei. We interpreted the taphonomic history to involve shrinkage of nucleoplasm, leaving a major part of the original nucleus volume molded by diagenetic void-filling apatite and the nucleoplasm forming a smaller globular body. Xiao *et al.* do not question our observations or our taphonomic analysis. A recent study (4) by two of the co-authors of Xiao *et al.* (1) even states that the nucleus-like bodies “may... topologically represent nuclei or other organelles” and that their presence falsifies the hypothesis that the Doushantuo fossils are giant bacteria (5, 6). Yet, in their comment on our paper, they conclude the opposite.

The first of their two arguments against the nucleus interpretation concerns crystal structure. Based on their previous taphonomic analyses

of Doushantuo fossils (7) they claim that cell walls and membranes “commonly” incorporate membrane-molding nanocrystals and that nuclear membranes should do so, too. However, that study concluded that membrane-addressed nanocrystals “exclusively occur in algal and acritarch fossils, but not in phosphatized animal embryo cells or embryonic envelopes” (i.e., the very fossils under discussion here). Walls and membranes of the latter are instead said to be “typically characterized by botryoidal and isopachous cements” [i.e., the fabric of void-filling apatite that also characterizes the molds of nuclei (2, 4)].

Minor differences in the crystallographic nature of the boundaries are in any case irrelevant in the context. Some factor must have created the parting surfaces that shaped the molds into their spitting images of nuclei. The original presence of a nuclear membrane, now only preserved as the surface of a mold, is so far the only reasonable hypothesis.

The other argument against the nucleus interpretation concerns size. The structures in the cleaving cells are said to be too large to be nuclei and the spores at release stage too small to host the full set of genetic material. Because eukaryotic nuclei in extreme cases may be up to 5 mm long (8) and our nondividing fossil nuclei are 44 to 106 μm in maximum diameter, the first objection is void. The second one seems to be based on a misconception that the size of the nuclei reflects the size of the genome. Nucleus size is mainly a function of cell size, however, and the karyoplasmic ratio (i.e., the volumetric ratio of nucleus versus cell) is remarkably stable in eukaryotic cells (9). Differences in cell (and therefore nucleus) size between taxa may indeed be related to genome size (10), but the karyoplasmic ratio is maintained nonetheless. Most important, the ratio is stable also in growing

cells (11, 12); thus, the same genome will be incorporated into nuclei of widely different sizes.

We interpreted the large cell size in the early cleavage stages of the Doushantuo fossils as the result of hypertrophic growth of the mother cell preceding encystment and palintomic cleavage; the large nucleus size is then a predictable result of hypertrophy. A modern analog (a parasitic dinoflagellate) shows hypertrophic growth with constant karyoplasmic ratio followed by palintomic cleavage resulting in spores an order of magnitude smaller than the nuclei of the late hypertrophic stages [figure 28 in (13)]. Xiao *et al.*'s conclusion that “either the nucleus interpretation or the ontogenetic connection must be incorrect” is therefore wrong.

Xiao *et al.*'s assertion that the inner cells of the “peanuts” are vegetative (i.e., not potentially gamete-forming) is both unsubstantiated and unfalsifiable. In most of our specimens, such as the one in figure 3, H to J, in (2), there is a clear diagenetic gradient where the innermost cells are not infilled and have their walls thickened by diagenetic cement. What we observed and claimed is that the peripheral cells are detached and form isolated structures that are consistent with a function as propagules. Xiao *et al.* dismiss these structures as taphonomic artifacts on the grounds that their appearance does not fully match that of the endospores of two modern mesomycetozoean taxa. This reflects their misconception that we based our reconstruction of the fossil life cycle on comparisons with those two modern taxa. Rather, our interpretation of the fossil life cycle was based on the intrinsic features of the fossils, where the identical envelope structure (2, 14–16) was a central piece of evidence that the fossils belong to the same organism; in fact, Xiao and colleagues have previously used this same criterion to assemble stages in the development of these same Doushantuo fossils (17, 18). We compared our observations with the very similar life cycles in modern protists, including both alveolates and mesomycetozoeans; however, we did not claim that the fossils are alveolates or mesomycetozoeans, but we noted that in both modern groups, as in the fossils, palintomic cleavage produces propagules, not multicellular bodies. In general appearance, the fossils may be most similar to modern mesomycetozoeans, but there are no synapomorphies that warrant a placement within that clade. Indeed, we concluded only that the fossils did not represent prokaryotes, crown metazoans, or multicellular stem-metazoans; we did not preclude a unicellular stem-metazoan affinity, but there is no evidence to substantiate such a placement.

Xiao *et al.* (1) seem to agree with us that at least some of the features used in support of animal affinity are holozoan symplesiomorphies. They insist, however, on invoking regular 2ⁿ cleavage and Y-shaped junctions between cleavage cells as animal characters, erroneously claiming that these do not occur in mesomycetozoeans

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and most other protists. Indeed, both these characters are known from, e.g., mononucleate mesomycetozoa (19). Early-branching holozoans have an animal-like genome that includes key elements of the genetic repertoire required for animal-grade multicellularity (20, 21). Evidently, the molecular machinery required for the formation of the Y-shaped cell junctions seen in the Doushantuo fossils evolved outside the metazoan total-group, indeed outside of opisthokonts, but was lost in Fungi and choanoflagellates (20). Similarly, the structure of the envelope is not a metazoan synapomorphy either, as we previously discussed (2).

Despite our differences, Xiao and colleagues are in close agreement with our general conclusions. This contrasts with common interpretations of the Doushantuo fossils as advanced metazoans [e.g., (16, 22–24)].

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25 January 2012; accepted 31 January 2012
10.1126/science.1219076

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In their comment on (1), Xiao *et al.* (2) included three claims that were not in the version we were given to respond to. The following is our further response:

Xiao *et al.* argued that “Only certain ciliates are known to have macronuclei approaching the size of nucleus-like structures in Doushantuo fossils..., but as Hultgren *et al.* were not arguing for a ciliate interpretation, the large size of the fossil microstructures requires another explanation.” We did refer to palintomy in ciliates in (1). Regardless, the size of the nucleus-like structures in the fossils is by no means extreme in eukaryotes. Nucleus size is mainly a function of cell size, and hypertrophic growth leading to giant cells typically entails giant nuclei in a variety of eukaryotes, both unicellular and multicellular (3–6). For example, *Xenopus* oocytes grow during oogenesis to a diameter of 1.2 to 1.3 mm and have a nucleus about 400 μm across (7). The size of the fossil structures is therefore consistent with nuclei.

They also claimed that, according to us, “[t]he purported nuclei...maintain a constant size through successive cell divisions...” We made no assertion of constant size. Our volumetric data are inconclusive with regard to the first few rounds of palintomic cleavage [figure S6 in (1)], but further cleavage must necessarily involve reduction of the nuclear volume, irrespective of whether development results in a multicellular body or a mass of propagules. The bodies in all respects appear and behave like diagenetically modified nuclei, and Xiao *et al.* (2) have not offered an alternative interpretation.

Finally, they suggested that movies we provided to them “...show that the purported endospores are attached to the thallus through cellular filaments and are indeed surrounded by faintly preserved thallus cells.” We show below (Fig. 1) a sequence of six tomographic slices through these structures. Patterns in the surrounding matrix (light gray fabric in the center of the images) suggest a cellular structure, as seen also in figure 3J in (1). This structure is, however, strongly degraded in comparison with the central cell mass (dark fabric to the left in the images) and the proposed propagules. We do not see any recurring features that could be interpreted as cellular filaments. Individual isolates wax and wane through the successive slices (examples shown by white arrows), with nothing attaching them to the central cell mass [“thallus” of Xiao *et al.* (2)]. This shows that they are floating in the matrix.

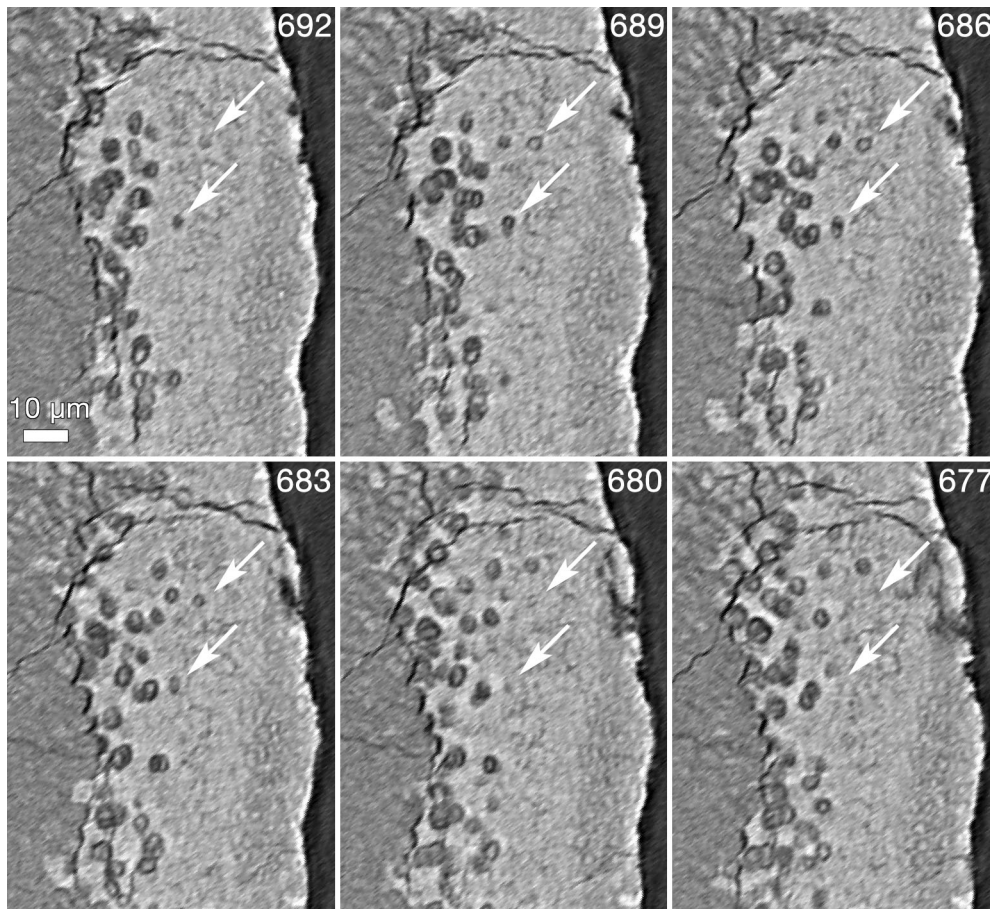


Fig. 1. Six tomographic slices through specimen SMNH X 4448 [also shown as figure 3, H to J, in (1)]. Darker tones mean lower x-ray attenuation. Numbers indicate slice number in tomogram (consecutive numbers are spaced at $0.37 \mu\text{m}$; thus, the figured slices are spaced $1.11 \mu\text{m}$ apart). The small, rounded aggregates (the positions of two of these through the sections are marked with white arrows) are interpreted as propagules/endospores becoming released from the central cell mass.

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Addendum received 4 May 2012; posted 22 May 2012.